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Method of analysis of chronic wounds

The present invention relates to methods of diagnosing susceptibility to chronic ulcers such as dermal ulcers, in particular chronic venous ulcers, arterial ulcers, diabetic ulcers and decubitus ulcers (pressure sores). These methods may also be used to predict the severity of ulcers and the efficacy of the healing response generated by the body.

The pathogenesis of chronic ulcers at present remains unknown, although many of the physiological mechanisms that initiate and cause persistence of ulcers have been studied closely. For example, reduced oxygen extraction, perivascular fibrin cuffing and trapping of cytokines are all observable features of venous ulcers. However, the link between these physiological disturbances and the pathogenesis of the condition remains elusive.

Venous ulceration alone costs the United Kingdom Health Service about £150 to £600 million each year and affects around 150,000 patients in the United Kingdom. Much of this cost is spent on care in the community, with up to 30% of community nursing time spent on treating leg ulcers. Some chronic ulcers respond rapidly to treatment, whilst others do not; indeed, many fail to heal over periods of several years. Furthermore, an ulcer may increase in size rapidly, or may remain static in terms of its size.

The choice of treatment should ideally be related to the prognosis. For example, if the prognosis is very good, then conservative treatment (such as on an out-patient basis) may be indicated. If the prognosis is poor, then a more interventional approach may be appropriate, involving surgery and skin grafting.

At present, there is no objective prognostic test for the severity of chronic ulcers, neither is there any way to estimate the likely time to healing. It is well known that many factors may influence the course of the disease, and at present it is upon these factors that clinicians and nurses subjectively judge the prognosis. These factors include the nature of the causative disease (for example diabetes, venous insufficiency, arterial insufficiency, ischaemia), patient age, nutritional status, ulcer duration, patient

compliance with treatment, the nature of the treatment, and other inexact criteria (Rijswijk, 1993; Skene *et al.*, 1992).

In many chronic inflammatory diseases, the up-regulation and/or dysregulation of cytokine production in inflamed tissue and wound fluid is thought to contribute both directly and/or indirectly to the pathology of the disease. Cytokines are peptide/protein immunomodulators that are produced by activated immune cells including thymus-derived T lymphocytes, B lymphocytes and monocyte/macrophages and may also be stored (e.g. in platelets) and synthesised by non-immune cells. The cytokines include interleukins, colony-stimulating factors for granulocytes and/or macrophages, tumour necrosis factors, and interferons.

Wound fluid (the exudate from wounds) contains a mixture of serum and tissue-derived proteins, including many cytokines. Its composition is thought to reflect the microenvironment of the wound site. This environment may be different within healing and non-healing chronic wounds; it has been postulated that chronic leg ulcers do not heal because there is a deficit of growth promoting cytokines (Schultz *et al.*, 1991). Conversely, a net excess of growth inhibiting cytokines may also be present; wound fluid from leg ulcers has been reported by several groups to inhibit fibroblast and keratinocyte proliferation (Bucalo *et al.*, 1989; Harris *et al*, 1991; Shakespeare *et al.*, 1991).

While transient inflammation is a key integral stimulatory process in the healing of acute wounds, excessive and prolonged inflammation can lead to tissue breakdown and can cause wound chronicity. In cutaneous animal models of inflammation, the response of the dermis to an intradermal injection of endotoxin (LPS) has been described. Neutrophil recruitment in LPS-induced injury was found to be associated with raised levels of TNFα, IL-1 and IL-8 (Silber *et al.*, 1994).

A recent report indicates that retention fluid from blisters of partial skin thickness burns, which contain relatively large amounts of cytokines and growth factors, have a surprisingly high level of IL-8 (Ono *et al.*, 1995). IL-8 is a potent chemoattractant for

neutrophils and there is convincing data which demonstrates that TNFα-induced transendothelial neutrophil migration is IL-8 dependent (Smart *et al.*, 1994).

The microvasculature of venous ulcers is characterised by pericapillary fibrin cuffs and by plugging of the capillaries by white blood cells. It has been shown that in patients with venous leg ulcers who display this pathological feature, the white blood cells express high levels of TNFα and the authors suggest that this may explain the absence of wound repair in these patients (Claudy *et al.*, 1991).

Two groups (Stacey *et al*, 1995; Harris *et al* (1995) have measured the levels of growth factors and cytokines in chronic leg ulcers from human patients and found that the levels of the inflammatory cytokines (IL-1, IL-6 and TNFα) were all significantly lower in wound fluid from the healing phase, when compared with the initial non-healing phase of the venous leg ulcers. Conversely, there was no alteration in the levels of growth factors such as PDGF, FGF and EGF.

Leg ulcers are also strongly associated with diabetic pathology. In diabetics, it seems that the mechanism of ulceration may be due to heightened levels of circulating inflammatory cytokines. In this respect, Foss *et al* (1992) have shown that serum TNFα levels are significantly higher in insulin-dependent type 1 diabetic patients than in non-diabetic controls. It has been postulated that raised levels of modified lipoproteins that are present in diabetics may stimulate macrophages to synthesise and release significantly higher levels of both IL-1 and TNFα than levels that are found in healthy patients. The release of cytokines from activated macrophages into the sub-endothelial spaces may have a significant role in the promoting the interaction of endothelial cells with mononuclear cells, so causing endothelial damage (Lopes-Virella, 1996).

The role of leukocytes in tissue damage in the liposclerotic skin of venous disease has also been investigated. In a significant number of patients, lipodermatosclerosis (LDS) is the prelude to or is associated with a venous leg ulcer. It has been shown that in severe LDS in the absence of a detectable venous leg ulcer, dermal staining from both IL-1 alpha and IL-1 beta is increased, which is thought to be a contributing factor in the observed progression to venous disease (Wilkinson *et al.*, 1993).

Patients with severe burn injuries are another patient group in which significant effort has been made to understand the contribution of pro-inflammatory cytokines to the healing process. Plasma levels of IL-1, TNFα and IL-6 have been monitored in burn patients throughout the healing process. The results indicate that the systemic cytokine response to burn injury is mainly represented by IL-6 (de Bandt *et al.*, 1994; Papini *et al.*, 1997). A similar study by Yamada *et al* (1996) measured blood levels of TNFα, IL-6 and IL-8. The increased levels of each cytokine were found to reflect the severity of the associated burn injury. In addition, the level of IL-1β has been positively correlated with burn size, thereby implicating this cytokine in the pathogenesis of thermal injury.

In addition to their role in orchestrating the host response to injury, cytokines such as TNFα, IL-1 and IL-6 are key regulators of matrix metalloproteinase and neutrophil elastase synthesis. There is a significant amount of data which suggests that raised levels of matrix metalloproteinases and neutrophil elastase are associated with poor wound healing, particularly in chronic skin wounds such as venous, diabetic, pressure ulcers and severe burn injuries. For instance, TNFα and elastase activity were found in the granulation tissue of venous stasis ulcers although these proteins are barely detectable in acute wounds (Claudy *et al.*, 1991; Wilkinson *et al.*, 1993). Grinnell and Zhu (1994; 1996) have also implicated neutrophil elastase in the delayed healing of chronic skin wounds.

It has also been demonstrated (Schultz *et al,* 1993) that the mitogenicity of fibroblast cultures in acute wound fluid is lost if the experiment is repeated using chronic wound fluid. Since the mitogenicity of chronic wound fluid could be restored in the presence of a protease inhibitor, this suggests that excess protease activity was responsible for the reduction in fibroblast activity.

The balance between matrix deposition and tissue turnover is fundamental in wound healing. It is thought that the balance between proteolytic enzymes and their natural inhibitors contributes to this. A recent report (Bullen *et al.*, 1995) has shown that chronic wounds contained significantly higher levels of gelatinases and the levels of

tissue inhibitors of metalloproteinase (TIMPs) were lower than in healing wounds. This data suggests that excess proteolysis in chronic wounds retards successful healing, and results from an imbalance of proteinase and inhibitors, as well as the presence of higher levels of activated metalloproteinases.

However, despite this fairly detailed knowledge that we now possess regarding the composition of the microenvironment of chronic ulcers, there is no practical test available which enables a clinician to judge the severity of such wounds or the probable success with which the ulcer is likely to heal without treatment. This is considered by the present inventors to be partially due to the marked genetic variation that exists at the multiple genetic loci that control the inflammatory and other immune responses that are involved with chronic ulcers.

Furthermore, currently, there is no way by which it can be predicted whether an individual is likely to be susceptible to chronic ulceration. There thus exists a great need for a reliable, objective test that would allow the identification of individuals who are at risk from contracting a chronic ulcer. Such a test would also be invaluable to allow the prognosis of ulcer severity and/or time to healing and would provide a clinician or nurse with an indication of what kind of treatment regime might be applicable in each case.

The inventors have noted an increased frequency of particular alleles in individuals in both population and family studies, in connection with the incidence of severe chronic ulcers that do not heal. It has been found that there is a link between the polymorphism type of various genes that encode inflammatory cytokines in a patient and the risk that the patient may develop a chronic ulcer. Furthermore, this association can be extended to allow diagnosis of the likely severity of a chronic ulcer, if already partially developed, and the prospective efficacy with which the ulcer will heal. Measurement of these polymorphisms can be made from small samples of patient's tissue, such as blood, and compared with a database of such polymorphisms for prognosis of the ulcer.

Summary of the invention

According to the present invention there is provided a method of determining susceptibility of a patient to developing a chronic ulcer, comprising determining the polymorphism type in genes that encode inflammatory cytokines in the patient. According to a second aspect of the invention there is provided a method of predicting the severity of a chronic ulcer in a patient comprising determining the polymorphism type in genes that encode inflammatory cytokines in the patient. According to a still further embodiment of the present invention there is provided a method of predicting the healing response in a chronic ulcer in a patient comprising determining the polymorphism type in genes that encode inflammatory cytokines in the patient.

Preferably, the chronic ulcer is a dermal ulcer, selected from the group consisting of chronic venous ulcers, pressure sores, decubitis ulcers, diabetic ulcers and chronic ulcers of unknown aetiology.

Polymorphisms are variants in the sequence of a gene within a population. Gene polymorphisms are therefore a mechanism by which individuals may exhibit variations within the range of what is considered to be biologically normal. They may be sequence alterations that are found in populations from different ethnic or geographic locations that, while having a different sequence, produce functionally equivalent gene products. A good example of such sequences are those of the major histocompatibility complex (MHC). Polymorphisms also encompass variations that can be classified as alleles and/or mutations that produce gene products which may have an altered function from that of the normal (wild type) gene product. Polymorphisms also encompass variations which can be classified as alleles and/or mutations which either produce no gene product, an inactive gene product or increased levels of gene product.

According to the present invention, it has been found that at various loci that encode genes for inflammatory cytokines, some allelic variants are over-represented in patients who suffer from chronic ulcers. It is these genetic polymorphisms that give altered

levels or activities of inflammatory cytokines that thus lead to an increased incidence of chronic ulcers, heightened severity and a decreased healing response in afflicted individuals. Such altered levels or activities may directly alter the microenvironment of an ulcer, or may exert downstream effects on molecules that themselves deteriorate the condition of the wound or impair its repair.

This discovery allows the early detection of a predisposition to developing a chronic ulcer and represents a much improved opportunity for medical intervention than treatment of the disease once the symptoms have already commenced. The supervision of a patient over a period of time in which he or she is thought to be at risk from developing a chronic ulcer then allows early diagnosis that may improve prognosis and allow preventative intervention before the clinical symptoms of the disease are noticed. This also means that patients who cannot be differentiated on the basis of their clinical symptoms may be separable on the basis of their genetic disposition to the disease; such analysis allows the development and application of more individual treatments that suit patients with subtle or undetectable differences in their disease state.

In most cases, the genetic polymorphisms that are associated with chronic ulcers cause an increase in the activity or levels of inflammatory cytokines. As discussed above, many research groups have previously attempted to find correlations between levels of inflammatory cytokines in various inflammatory conditions, but no real consensus has emerged as to which cytokines are causative and which are simply the result of increased levels or activities of other cytokines. It is therefore hypothesised that this failure is due to the fact that it is subtle alterations in the activities of inflammatory cytokines that are responsible for changes in patterns of susceptibility to and prognosis of chronic ulcers.

The polymorphisms that are the subject of the present invention are present in any inflammatory cytokine whose activity is altered in the microenvironment of chronic ulcers. Preferably, the polymorphisms are present in the inflammatory cytokines IL-1, IL-6, IL-8 and TNFα, although other suitable candidates will be apparent to those of skill in the art.

Of particular suitability for use in accordance with the present invention are the polymorphisms listed below, which are indicative of increased risk/severity of developing a chronic ulcer.

There are three known IL-1 genes, that form a cluster on human chromosome 2q13. IL-1A and IL-1B produce IL-1α and IL-1β, respectively. IL-1RA binds to IL-1 receptors and acts as a receptor antagonist. The presence of allele 2 of the IL-1A -889 polymorphism or allele 2 of the +3953 polymorphism of the IL-1B gene is a positive indicator of susceptibility to chronic ulcers. This is thought to be due to an elevation of active levels of IL-1 produced by monocytes in individuals that possess these polymorphisms. Individuals that are heterozygous for either of these polymorphisms are at greater risk than those individuals that possess wild type IL-1A or IL-1B loci. Homozygous individuals are at even greater risk. Those individuals who possess both polymorphisms, and are either heterozygous or homozygous for either or both of these polymorphisms are at greatest risk.

A further IL-1B polymorphism herein linked to chronic ulcers is the IL-1B –511 polymorphism. Details of other polymorphic sites in IL-1 genes may be found in the following references: Laurent *et al.*, 1997; Heresbach et al, 1997; Tarnow *et al.*, 1997a; Tarnow *et al.*, 1997b; Cork *et al.*, 1996; Guasch *et al.*, 1996; Clay *et al.*, 1996; Lakemore *et al.*, 1996; Satsangi *et al.*, 1996; Bioque *et al.*, 1995; Crusius *et al.*, 1995; Danis *et al.*, 1995b; van den Veldan *et al.*, 1993; Bailly *et al.*, 1993; Feltes *et al.*, 1993; Jacob *et al.*, 1993; di Giovine *et al.*, 1993; Todd *et al.*, 1993 and Richter *et al.*, 1989.

In transgenic mice that over-produce TNFα, abnormal TNFα production has been shown to contribute to disease initiation and progression of rheumatoid arthritis, systemic inflammatory response syndrome and diabetes (Probert *et al.*, 1996 *J Leukocyte Biol* **59(4)**: 518-525). TNFα is another inflammatory cytokine for which polymorphisms that generate altered activity from normal are herein linked with chronic ulcers, particularly chronic ulcers. An example of such a polymorphism is that at position –308 in the TNFα gene. Further examples of TNFα polymorphisms which the skilled man will be able to apply to the diagnosis of chronic ulcers may be found in the following references: Abraham *et al.*, 1993; Wilson *et al.*, 1992; Pociot *et al.*, 1991;

Seitzer *et al.*, 1997; Brinkman *et al.*, 1997; Demeter *et al.*, 1997; Louis *et al.*, 1996; Bouma *et al.*, 1996; Chen *et al.*, 1996; Fong *et al.*, 1996; Wilson *et al.*, 1995; Danis *et al.*, 1995a; Verjans *et al.*, 1994 and Stokkers *et al.*, 1995.

The gene that encodes IL-6 also contains polymorphisms whose presence can be positively correlated with susceptibility to chronic ulcers. One example is the *Bgl*II mutation (Blankenstein *et al.*, 1989; Fugger *et al.*, 1989a). Further examples may be found in the following references: Murray *et al.*, 1997; Danis *et al.*, 1995a; Stokkers *et al.*, 1995; Toungouz *et al.*, 1994; Shalhevet *et al.*, 1993; Jacob *et al.*, 1993; Titenko *et al.*, 1991; Fugger *et al.*, 1989b and Dawson *et al.*, 1993.

With reference to IL-8, the *Hind*III polymorphism is of use in the diagnosis of susceptibility to chronic ulcers (Fey *et al.*, 1993).

Polymorphisms may also be present in genes that encode receptors for inflammatory cytokines, whose activity is necessary for the effective biological function of the cytokine. Examples of such polymorphisms are the promoter polymorphism of the plasminogen activator inhibitor (PAI-1) gene that causes an altered response to IL-1 (Dawson *et al.*, 1993) and the polymorphisms that are responsible for alternative forms of the human granulocyte colony stimulating factor (G-CSF) that cause changes in growth signal transduction (Ziegler *et al.*, 1991).

Typing of the genetic polymorphisms of a patient are carried out *ex vivo*. Assessment of polymorphism type may be either through the use of specific antibodies directed against the antigenic determinants of the inflammatory cytokines or may be by analysis of the genotype of the patient. Preferably, typing is by genetic analysis of the inflammatory cytokine locus.

In order to ascertain the genotype of a patient, a sample of the DNA of that patient must be available. This sample may be obtained from any tissue of the body. Commonly-used tissues for biopsy are the blood, buccal epithelium, skin or hair. Preferably, the DNA sample is obtained from blood samples. In a preferred embodiment, the DNA is obtained from blood cells obtained from a finger prick of a patient. The blood may be

collected on absorbent paper, or preferably on an AmpliCard™ (University of Sheffield, Department of Medicine and Pharmacology, Royal Hallamshire Hospital, Sheffield, England S10 2JF), also described in Tarlow JW. *et al.* 1994 *Journal of Investigative Dermatology*: **103**: pp387-389.

This embodiment has the advantage of requiring only a small amount of blood and avoids the necessity for venipuncture or a tissue biopsy. However, other means for collecting DNA and determining polymorphism patterns as known in the art can be used.

Molecular DNA typing of the inflammatory cytokine gene locus may be carried out by detection and assignation of the DNA polymorphisms in the inflammatory cytokine gene through the use of various techniques that will be well known to those of skill in the art. There are three preferred methods. These are first the detection of restriction fragment length polymorphisms (RFLPs); second, Southern blotting of PCR-amplified DNA using specific probes; and third, direct sequencing of PCR products. The latter method, which although more laborious is more stringent, is generally the preferred method of the present invention.

RFLPs are changes in a specific DNA (termed a polymorphism if the differences between human individuals occur more frequently than every 10^7 bases) that may be traced using restriction enzymes. When a polymorphism occurs in a consensus sequence that is recognised by a particular restriction enzyme so that this sequence is no longer recognised, the DNA fragments produced by restriction enzyme digestion will be of different sizes. The various possible fragment sizes from a given region therefore depend on the precise sequence of the DNA in the region. This variation in the fragment sizes is termed a restriction fragment length polymorphism (RFLP), and can be visualised by separating the DNA according to its size on an agarose gel.

The individual fragments may be visualised by annealing to a labelled oligonucleotide probe that is specific for the sequence of the fragment of interest. Various methods of labelling the probe will be known by those of skill in the art and will most commonly involve the use of radioactivity or fluorescent or enzymatic tags.

According to the present invention, the more preferred method of detection of polymorphisms is through the amplification of a DNA fragment that is then analysed using probes that are specific for the particular polymorphism of interest. Alternatively the amplified DNA fragment may be sequenced directly. Preferably, the DNA fragment is amplified using the polymerase chain reaction (PCR). The amplified DNA fragment will of course comprise the portion of the inflammatory cytokine gene that contains the polymorphism of interest.

A diagnostic length of DNA may be amplified by PCR using primers raised to conserved DNA sequence in the inflammatory cytokine gene. By a diagnostic length is meant a fragment of sufficient length to allow discernment of the characterising polymorphisms of each inflammatory cytokine antigen type. Thus, the fragment must be of sufficient length to allow an oligonucleotide primer to hybridise specifically with this sequence. As will be apparent to those of skill in the art, this fragment of DNA is of at least 50 bases, preferably 100 bases, and most preferably more than 400 bases in length.

The primers used to amplify the DNA fragment may be designed by anyone of skill in the art so as to be complementary in sequence to the gene sequence that flanks the polymorphism. Preferably the reaction conditions for PCR are as described herein or in Kimura and Sasazuki, 1992.

The PCR product can be purified and immobilised for hybridisation by methods commonly used in the art. The fragment may be purified by submarine gel electrophoresis and immobilised on membranes (Boehringer) as described in Kimura and Sasazuki, 1992.

For analysis by Southern blotting, the purified and immobilised PCR product is challenged with labelled sequence-specific probes. Each specific probe comprises an oligonucleotide of complementary sequence to the particular defining polymorphic region of the inflammatory cytokine locus. These probes are specific for each inflammatory cytokine polymorphism type. Under conditions of a certain stringency, each oligonucleotide will only hybridise to the polymorphic DNA sequence against which it is raised and thus will provide polymorphism typing with much more accuracy than is possible using serological

methods. The conditions of stringency to use will be facile for the man of skill in the art to ascertain (see, for example Sambrook *et* al., 1989; Molecular Cloning: a laboratory manual; Ausubel *et* al., eds., John Wiley & Sons. 1992). A further probe capable of specific binding to all wild type loci may be used as a control.

The method of detection of bound probes may be by any one of the methods commonly used in the art. Preferably the probes themselves are labelled, either by radiolabelling, or by chemical modification, for example using digoxigenin (Kimura and Sasazuki, 1992; Boehringer Mannheim catalogue). Detection may be by autoradiography, or by chemiluminescence, respectively, depending on the system chosen. Most preferably, the invention uses digoxigenin-labelled oligonucleotides.

When using digoxigenin-labelled oligonucleotides, a labelled anti-digoxigenin antibody-enzyme conjugate is used for the detection of oligonucleotide. This specific reaction can be visualised by chemiluminescent detection using an AMPPD substrate in accordance with the manufacturer's instructions (Boehringer Mannheim). In the preferred embodiment of the invention, the conjugated enzyme comprises an alkaline phosphatase conjugate.

A preferred method of detection is by direct sequencing of the PCR products. This method is commonplace and will be well-known to those of skill in the art. Briefly, the initial PCR product is subjected to a second amplification employing an Applied Biosystems sequencing kit, as described in Morrison *et al.* 1993. The product is purified twice using phenol/chloroform and then precipitated using ethanol. For the sequencing reaction, the DNA is loaded onto a 6% polyacrylamide gel, before direct sequencing is performed in both forward and reverse directions (in triplicate) using fluorescence-labelled dideoxynucleotide termination on an Applied Biosystem 373A Automated DNA Sequencer. Alternative sequencing kits, PCR purification kits and automated sequencers are readily commercially available and may be employed in the present invention.

According to a further aspect of the present invention, there is provided a diagnostic kit for typing of the polymorphism type of an inflammatory cytokine locus in a patient. All three detection methods described above lend themselves readily to the formulation of kits that can be used in diagnosis. Such kits will contain reagents suitable for applying the method

of the invention to detect the appropriate polymorphisms and will thus provide the necessary materials to carry out the molecular biological reactions that are described above. These are packaged into suitable containers or supports useful for performing the assay.

The essential components of the assay vary depending upon which embodiment of the invention is to be utilised. Regarding the detection of RFLPs, the essential components of the assay include the restriction enzyme associated with the polymorphism and the specific probe. Additionally, packages containing concentrated forms of reagents and buffers used for hybridisation, prehybridisation, DNA extraction and the like may be included. In particular however, labelled probe, or reagents suitable to form conveniently labelled probe are useful in facilitating the conduct of this method of the invention.

In connection with the amplification of DNA fragments using PCR and their subsequent analysis using specific probes, the essential components of the assay kit will include the thermostable DNA polymerase enzyme associated with amplification of the DNA fragment and a suitable probe. For direct sequencing of PCR products, the essential components are the specific primers, a suitable thermostable DNA polymerase enzyme, ATP, the mixed nucleotide units for extension of the nucleotide chain, and fluorescent-labelled dideoxynucleotide termination products.

All documents mentioned in the text are incorporated herein by reference.

Various aspects and embodiments of the present invention will now be described by way of example and illustrated with reference to the figures. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

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EXAMPLES

Analysis of genetic polymorphisms

The subject's finger was cleaned with antiseptic wipes and the skin was punctured with a sterile lancet. Finger-stick blood samples were collected on DNAase-free blotting paper (Tarlow *et al.* 1994) and analysed blind for polymorphism in the IL-lA gene at position -889 (McDowell *et al.* 1995), in the IL-1B gene at positions –511 (Di Giovine *et al.*1992) and +3953 (Di Giovine *et al.* 1996), the IL-1RA gene intron-Z (Tarlow *et al.* 1993), and the TNFA gene at position –308 (Wilson *et al.* 1992).

A reaction mix excluding Taq polymerase was prepared and 1 mm^2 dried blood spots were added prior to heating at 95°C for 15 min. Taq polymerase (1.25 u. GibcoBRL-UK) was then added and PCR started. All reactions were carried out in 20 mM TrisHCl, 50 mM KCl. 0.2 mM each dNTP and 0.05% W-1 detergent. The $MgCl_2$ and printer concentrations varied in each type of reaction and are detailed below.

Analysis for TNFα

The single G/A base variation polymorphism at –308 in the TNFα gene sequence was identified by PCR amplification of genomic templates. A single base mismatch was incorporated into one of the primers in order to complete a *Nco*I restriction site.

Primer 1: AGG CAA TAG GTT TTG AGG GGC AT

Primer 2: TCC TCC CTG CTC CGA TTC CG

PCR conditions were as follows:

Final concentration of primers: 2μM.

1.5mM MgCl2 was used throughout the reactions.

1 cycle [94° (3 minutes); 60°C (1 minute); 72°C (1 minute)];

35 cycles [94° (1 minute); 60°C (1 minute); 72°C (1 minute)];

1 cycle [94° (1 minute); 60°C (1 minute); 72°C (1 minute)].

Restriction enzyme digestion used 6 units per 30µl reaction mixture of NcoI at 37°C for 8 hours. Sizing was using 2% agarose gels or 8% SDS-PAGE (Laemmli, 1970).

Allele 1 yields 2 fragments of 87bp and 20bp.
Allele 2 contains no *NcoI* site and is thus not digested. Consequently, this allele only yields one 107bp product.

Analysis for IL-1

IL-1A –889

Primer 1: AAG CTT GTT CTA CCA CCT GAA CTA GGC
Primer 2: TTA CAT ATG AGC CTT CCA TG

Final concentration of primers: 0.8 µM;
1mM $MgCL_2$ used through out the reactions.
1 cycle [96'C (2 min);
45 cycles [94°C for 1 min; 50°C for 1min; 72°C for 1 min; 50°C for 1 min.

PCR product is digested overnight at 37°C with 6 units per 30µl reaction of *NcoI*. and restriction pattern visualised by electrophoresis through a 6% PAGE (1.50V for 2.5 hours). This gave products of 83bp+16bp (allele 1) and 99bp (allele 2).

IL-1β –511;

Primer 1: TGG CAT TGA TCT GGT TCA T
Primer 2: GTT TAG GAA TCT TCC CAC TT

Final concentration of primers was 1 µM.
25mM $MgCl_2$ was used throughout the reaction.

1 cycle [95°C for 2 min; 53°C for 1 min; 74°C for 1 min]
35 cycles [95°C for 1 min; 53°C for 1 min; 74°C for 1 min]

Digestion of products was with 3 units *Avo*I per 30µl reaction at 37°C overnight. yields products of 190bp+ 114bp (allele 1) or 304bp (allele 2).

IL-1B + 3953:

Primer 1: CTC AGG TGT CCT CGA AGA ATC AAA
Primer 2: CCT TTT TTG CTG TGA GTC CCG

Final concentrations of primers: 2 µM.
2.5 mM $MgCl_2$ was used throughout the reactions.

35 cycles [95°C for 2 min; 67.5°C for 1 min; 74°C for 1 min]
3 cycles [95°C for 1 min; 67.5°C for 1 min; 74°C for 5 min]

The PCR products were digested with 10 units per 30µl reaction of *Taq*I at 65°C overnight. The resulting products of 12bp + 83bp + 97bp (allele 1) and 12bp+ 182bp (allele 2) are diagnostic.

IL-IRN (intron 2)

VNTR;
Primer 1: CTC AGC AAC ACT CCT AT
Primer 2: TCC TGG TCT GCA GGT AA

Primer concentrations were 1µM.
1.75 mM $MgCl_2$ was used throughout the reactions.

1 cycle [95°C for 1 min]
35 cycles [94°C for 1 min; 60°C for 1 min; 70°C for 2 min]
1 cycle [70°C for 5 mins; 55°C for 5 min]

Electrophoresis in agarose was performed at 90V for 45 min. Allele 1 (4 repeats) was 412bp; allele 2 (2 repeats), 240bp; allele 3 (3 repeats), 326bp; allele 4 (5 repeats), 498bp; and allele 5 (6 repeats), 584bp.

All PCT products were stained with ethidium bromide 0.2μg/ml and visualised under ultraviolet light following electrophoresis. All PCR screening methods used in this study have been extensively validated.

Statistical methods

Data analyses should be performed as follows. The presence or absence of a copy of the less frequent allele for each DNA polymorphism or the presence or absence of a composite genotype formed by combining pairs of DNA polymorphism in the IL-1 gene cluster will be compared with the presence or absence of chronic dermal ulceration. This will involve logistic regression analysis and the calculation of odds ratios with the appropriate confidence interval. The strength of association will be assessed by the χ^2 test or Fisher's exact test. A Bonferroni correction (Miller, 1981) will be applied to account for multiple comparisons. All analyses may be performed with the SAS statistical package.

A similar analysis should be performed looking at the severity (defined on a categorical scale) of the ulceration.